Interaction of a 5-*trans*-Vinylcarboxylic Acid Analogue of Pyridoxal 5′-Phosphate with Apoaspartate Aminotransferase. Covalent Labeling of the Enzyme†

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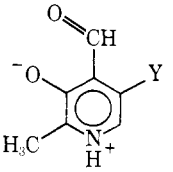
ABSTRACT: The 5-*trans*-vinylcarboxylic acid analogue of pyridoxal 5′-phosphate has been prepared. Its pK_a values were determined as 3.08, 4.10, and 7.33. The third pK_a, that of the pyridinium nitrogen, is considerably lower than that of 8.2 observed for the corresponding saturated compound, 5′-carboxymethyl-5′-deoxypyridoxal. Absorption spectra of individual ionic forms have been resolved into component bands using lognormal distribution curves. The vinylcarboxylic acid analogue inactivates apoaspartate aminotransferase slowly at pH 8.3. An initial product absorbs at 26 kK (385 nm) and is converted slowly to a species with a narrow absorption band at 24.0 kK (417 nm). Meanwhile, the circular dichroism in the same region changes from positive to negative. At pH 5.2 the product absorbs at 25.2 kK (397 nm). The 24.0-kK (417 nm) form is not reducible with sodium borohydride and the tightly bound chromophore is not released from the protein during denaturation by acid, base, or heat. L-Glutamate and *erythro*-β-hydroxyaspartate both facilitate the formation of the 24.0-kK form. The reaction of the analogue with apoenzyme in the presence of *erythro*-β-hydroxyaspartate is also accompanied by transient peaks, presumably representing quinonoid forms, at 19.0 kK (526 nm) and 20.3 kK (492 nm). The analogue reacts at basic pH with arginine, α-amino-γ-guanidinobutyric acid, ornithine, cysteine, α,γ-diaminobutyric acid, ethylenediamine, and 1,3-diaminopropane to yield products with narrow absorption bands centered in the 24.0–24.4-kK (417–410 nm) region and resembling the product formed with the apoenzyme. Nuclear magnetic resonance and absorption spectroscopy indicate that the reaction with α,γ-diaminobutyric acid proceeds via a hexahydropyrimidine derivative to a substituted tetrahydropyrimidine (a cyclic Schiff base) which is the final product. A similar reaction sequence with the apoenzyme is postulated and a structure with an unknown X group from the enzyme replacing the γ-amino group of α,γ-diaminobutyric acid is proposed for the 24.0-kK (417 nm) chromophore obtained with the apoenzyme. The proposed reactions are closely related to enzymatic and nonenzymatic reactions of pyridoxal 5′-sulfate (Yang, I.-Y., Khomutov, R. M., and Metzler, D. E. (1974), *Biochemistry 13,* 3877).

Although the function of pyridoxal phosphate (pyridoxal-P, I)[1] in transamination and related reactions has been studied extensively, the role of the 5′-phosphate group of the coenzyme is not fully understood. Investigations with 5′-modified analogues of pyridoxal-P may clarify the function of this phosphate group and may provide clues to understanding the participation of functional groups of the protein in catalysis.



I, Y = $-CH_2OPO_3^{2-}$, pyridoxal-P
II, Y = $-CH_2CH_2COO^-$
III, Y = $-CH{=}CH-COO^-$ (*trans*)
Y = $-CH_2OSO_3^-$, pyridoxal 5′-sulfate

The 5-propionic acid analogue (5′-deoxy-5′-carboxymethylpyridoxal, II, Iwata and Metzler, 1967) has been tested with aspartate apoaminotransferase (apoAAT, Furbish et al., 1969), D-serine apodehydratase (Dowhan and Snell, 1970), glutamate apodecarboxylase (Mekhanik et al., 1972), and apophosphorylase (Graves and Wang, 1972). In all cases compound II displayed little or no activity as a coenzyme. With apoAAT it binds in a normal way to form an "internal" Schiff base but it is less than 0.2% as active as pyridoxal-P. When a large excess of the amino form of II was added the activity increased to 1%.

The corresponding unsaturated *trans*-vinylcarboxylic acid analogue (*trans*-5′-deoxy-5′-carboxymethylenepyridoxal, III) has been synthesized (Hullar, 1969; Kolobushkina et al., 1974) and has been tested with apoAAT (Tumanyan et al., 1974), glutamate decarboxylase (Mekhanik et al., 1972), and tyrosine decarboxylase (Hullar, 1969). It was reported to bind to apoAAT at pH 5.2, but the resulting complex was unstable. In this paper we report the irreversible covalent binding of III to apoAAT. A modified synthesis of III together with some physicochemical properties are also described.

Materials and Methods

trans-5′-Deoxy-5′-carboxymethylenepyridoxal (III). To a solution of *trans*-5′-deoxy-5′-carboxymethylenepyridoxine (IV) (Kolobushkina et al., 1974) (1.63 g) in tetrahydrofuran (250 ml) and water (10 ml) was added 5 g of active

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[1] Abbreviations used are: pyridoxal-P, pyridoxal 5′-phosphate; AAT, aspartate aminotransferase; kK, kilokaysers; 1 kK = 10^3 cm^{-1}; $\tilde{\nu}$, wavenumber; $\tilde{\nu}$ (kK) = $10^4/\lambda$ (nm).

Table I: Parameters for the Lowest Energy Absorption Bands of Compounds III and IV.[a]

Compound and Ionic Form	Position ν_0 (kK) (nm)	Height $\epsilon_0 \times 10^{-3}$ (l. mol^{-1} cm^{-1})	Width W (kK)	Skewness ρ	Molar Area[b] a^0 (km mol^{-1})	Fraction[b] of Subform, f_i
III aldehyde						
H_3P	28.57 (350)	1.29	4.57	1.18	(63.1) 334	0.189
HP (±)	25.00 (400)	3.18	4.00[c]	1.36[c]	(138) 468[d]	0.295
HP (0)	27.28 (367)	2.07	4.70[c]	1.32[c]	(105) 273	0.385
P	24.69 (405)	6.95	4.48	1.34	(336) 364	0.924
hydrate						
H_3P	32.47 (308)	8.01	3.77	1.36	(327) 403[d]	0.811
HP (±)	29.80[c] (336)	1.72	3.95[c]	1.37[c]	(73.7) 422[d]	0.175
HP (0)	33.14 (302)	1.19	4.13	1.36[c]	(53.5) 369[d]	0.145
P	30.06 (333)	0.60	3.56[c]	1.36[c]	(23.2) 304[d]	0.076
IV H_3P	32.78 (305)	9.93	3.76	1.30	403	1.00
HP (±)	29.67 (337)	6.14	3.96	1.34	(263) 422[d]	0.623
HP (0)	33.71 (297)	3.10	4.13	1.36	(139) 369	0.377
P	29.89 (335)	6.61	4.32	1.02	304	1.00

[a] Band positions and widths are given in kilokaysers (kK); $\tilde{\nu}$ (kK) = $10^4/\lambda$ (nm). The parameters $\tilde{\nu}_0$, ϵ_0, W, and ρ are defined by Metzler et al. (1973). [b] Measured areas a, when not equal to molar areas a^0, are placed in parentheses. For a given subform, e.g., cationic free aldehyde, $a^0 = a_i/f_i$, where f_i is the fraction of a given subform. For a given ionic form, e.g. H_3P, $\Sigma_i f_i = 1$. [c] Parameters fixed at preselected values. [d] The molar area for the dipolar ionic of alcohol IV was assumed to be 1.21 times that of pyridoxine (the area of the cation is 1.31 and that of the anion 1.12 times those of the corresponding forms of pyridoxine). Molar areas of hydrate forms were assumed identical with those of corresponding forms of the alcohol IV. This permitted calculation of the fraction of hydrate and aldehyde for each form and the a^0 values for the aldehyde forms. For the HP form a^0 of the dipolar ion was assumed equal to 1.17 times that of the average value (400 km mol^{-1}) observed for other aldehydes, just as is observed for the anion (see Harris et al., 1975).

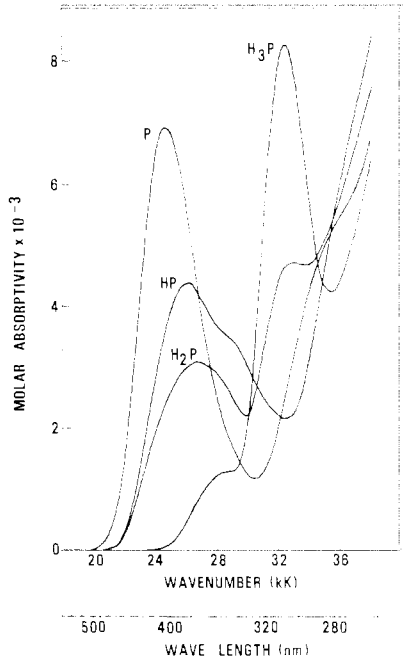


FIGURE 1: Molar absorptivities of the four ionic forms of compound III. The ionic forms are designated H_3P, H_2P, HP, and P according to the extent of protonation.

manganese dioxide (Mancera et al., 1953; Harfenist et al., 1954). As the product (III) is remarkably photosensitive, care was taken not to expose the reaction mixture to light. The mixture was stirred at room temperature for 1.5 hr, at which time the absorbance ratio, $A_{24.7\,kK}/A_{32.4\,kK}$ ($A_{405\,nm}/A_{309\,nm}$), attained its maximum. The reaction mixture was filtered and the filtrate was evaporated in vacuo to dryness. Recrystallization of the residue from methyl ethyl ketone gave III (0.91 g, 64%). The product decomposed at about 200°. Nuclear magnetic resonance (NMR) (4% NaOD/D_2O, Tier's salt as internal standard) δ 2.36 ppm (s, 3 H), 6.24 (d, 1 H, J = 16 Hz), 7.45 (s, 1 H), 7.80 (d, 1 H, J = 16 Hz), 10.15 (s, 1 H). Anal. Calcd for $C_{10}H_9NO_4$: C, 57.97; H, 4.38; N, 6.76, Found: C, 57.89; H, 4.66; N, 6.56. For spectroscopic titration and critical experiments with the enzyme, compound III (100 mg), as obtained above, was further purified on an Amberlite CG 50 column (H^+ form, 1.6 × 35 cm). Water was used for elution. Fractions with absorbance ratio ($A_{24.7\,kK}/A_{32.4\,kK}$ at pH 9.4) greater than 2.7 were pooled, evaporated, and lyophilized; yield, 78 mg. Electronic spectra are shown in Figure 1 and resolved absorption bands for aldehyde and covalent hydrate forms are given in Table I.

Enzyme. Aspartate aminotransferase was prepared from pig hearts following the procedure of Jenkins et al. (1959), with a modification by Martinez-Carrion et al. (1967). This is the α subform of the cytoplasmic enzyme. The absorbance ratio ($A_{23.3\,kK}/A_{29.4\,kK}$, pH 4.7–4.8) was in the range of 3.5 to 4.5. Determination of the enzyme concentration was based upon molar absorptivities of 6.36×10^4 and 6.55×10^4 at 35.8 kK for the apoenzyme and holoenzyme, respectively (Furbish et al., 1968), and a molecular weight of 4.63×10^4 (Ovchinnikov et al., 1973). The direct method of Jenkins (Jenkins et al., 1959; Furbish et al., 1968) was employed for measurement of enzyme activity.

Apoaspartate aminotransferase was prepared following the method of Scardi et al. (1963) with an adaptation by Furbish et al. (1968). The apoenzyme thus prepared had no detectable activity and was reactivated to 85–96% of that of the native enzyme when reconstituted with pyridoxal-P in tenfold excess.

Spectra. Absorption spectra were measured with a Cary Model 1501 recording spectrophotometer equipped with a Cary-Datex digital output system and an IBM card punch. Spectra were corrected for baseline errors and in some cases for small amounts of turbidity and were replotted automatically as molar absorptivity vs. wavenumber ($\tilde{\nu}$) at the Iowa State University Computation Center. Spectrophotometric titration as described by Nagano and Metzler (1967) and by Johnson and Metzler (1970) coupled with the method of Sims (1926) was used to determine the pK_a values of III.

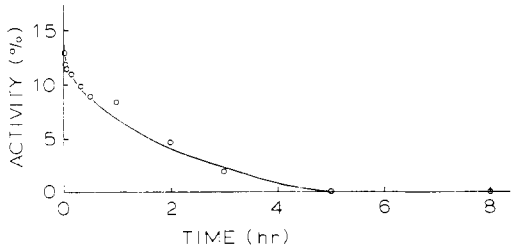


FIGURE 2: Inactivation of apoAAT by compound III. ApoAAT was incubated with compound III at 25°. Aliquots were taken from the mixture at different times and pyridoxal-P was added and the activity was measured (see text). Activity, as a percentage of that of apoAAT reconstituted with pyridoxal-P, was plotted against the incubation time.

Circular dichroism spectra were taken with a JASCO Model ORD/UV-5 spectrophotometer equipped with Sproul Scientific SS-20-2 modification. Hitachi-Perkin-Elmer R20B and Varian A-60 nuclear magnetic resonance spectrometers were used. Elemental analysis was performed by Chemalytics, Inc.

Results

The calculated absorption spectra of the four ionic forms of III are shown in Figure 1. These forms are related by the values of pK_a of 3.08, 4.10, and 7.33 which may be compared with the values of 3.45, 4.56, and 8.22 for the saturated acid II and 3.62, 6.10, and 8.33 for pyridoxal-P. The absorption spectra of the cationic (H_3P), dipolar ionic (HP), and anionic (P) forms of III were further resolved using lognormal distribution curves (Johnson and Metzler, 1970; Metzler et al., 1973; Harris et al., 1975). The band parameters thus obtained are summarized in Table I.

The spectra of the corresponding 4′-alcohol (IV) were also recorded and pK_a values of 4.94 and 8.86 were evaluated. The excellent fit of a theoretical curve based on these values to experimental spectrophotometric titration curves indicates that it is not necessary to take into account any influence of the dissociation of the carboxylate group in evaluating these two pK_a's. Spectra of the ionic forms of IV are also given in Table I.

Activity of the Complex of III and ApoAAT. Compound III (1.01×10^{-3} *M*) was incubated with apoAAT (1.02×10^{-4} *M*) at 25° in 0.07 *M* triethanolamine buffer (pH 8.3). The mixture was diluted 300 times and assayed for activity. No activity was detected with incubation times from 30 sec to 20 min.

Inactivation of ApoAAT with III. A mixture of III (8.30×10^{-4} *M*) and apoAAT (7.65×10^{-5} *M*) was incubated at 25° in 0.1 *M* triethanolamine buffer (pH 8.3). Ten-microliter aliquots were taken from the mixture at different times and incubated with 5-μl portions of pyridoxal-P (2.16×10^{-3} *M*) at 25° in 0.1 *M* triethanolamine buffer (pH 8.3) for 10 min. After being diluted 200 times, the mixtures were assayed for activity. Results are summarized in Figure 2. The enzyme lost 85% of its activity immediately and was totally inactive after 5 hr of incubation.

Reaction of III with ApoAAT. The apoenzyme was incubated with III in a cuvette at 10° in 0.1 *M* triethanolamine buffer (pH 8.3) with final concentrations of 131 and 87.1 μ*M* for apoAAT and III, respectively. The reaction was followed by measuring the absorption spectrum at different times. Immediately after mixing a band appeared at 26.0 kK (385 nm), then shifted to about 25 kK (400 nm) in 30 min. This 25-kK peak further shifted over a period of about

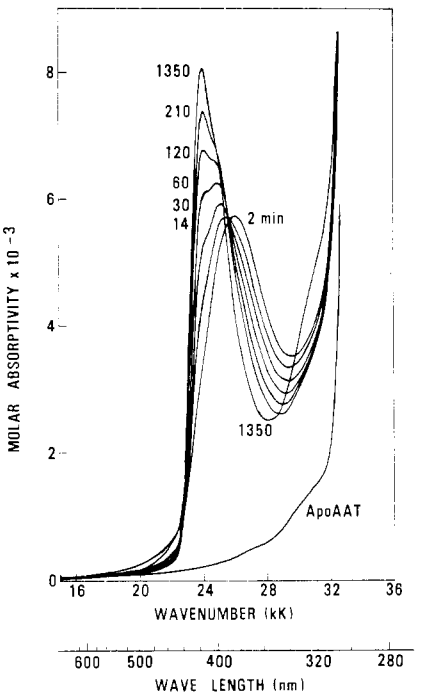


FIGURE 3: The reaction of apoAAT with compound III followed spectrophotometrically. The numbers designate the times in minutes following the mixing of the two components. The spectrum of apoAAT before the addition of III is also shown.

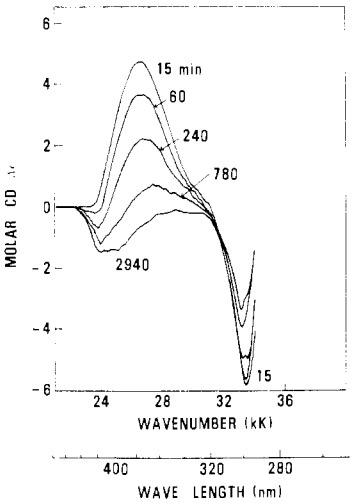


FIGURE 4: The reaction of apoAAT with compound III at 10° followed with circular dichroism in 0.1 *M* triethanolamine buffer (pH 8.3). The numbers are the time in minutes following the mixing of the two components. Final concentrations were: apoAAT, 131 μ*M*; III, 87.1 μ*M*.

20 hr to a final band centered at 24.0 kK (417 nm) with an increased intensity, a decreased band width and a shoulder on its higher energy side (Figure 3). After the 24.0-kK band had reached its maximum, it decayed very slowly. These spectral changes are paralleled by those in circular dichroism (Figure 4). The CD initially was positive at 26.6 kK (376 nm), suggesting that the initial product is a normal dipolar ionic Schiff base with the ε-amino group of lysine-258 (Ovchinnikov et al., 1973). The final 24.0-kK (417 nm) absorption band is associated with a negative CD. The final product also has a bright blue fluorescence under ultraviolet light. These characteristics are similar to those of the complex of pyridoxal 5′-sulfate with apoAAT (Yang et al.,

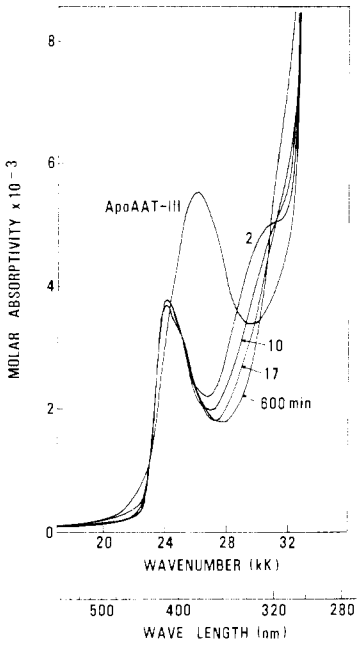


FIGURE 5: Treatment of the 26.0-kK (385 nm) form of apoAAT-III with L-glutamate. ApoAAT (103 μM) and compound III (72.9 μM) were incubated at 27° in 0.1 M triethanolamine buffer (pH 8.3). After 4 min of incubation L-glutamate (pH 8.3) was added. Final concentrations were: apoAAT, 93.6 μM; III, 66.4 μM; L-glutamate, 17.7 mM. The numbers are the times in minutes after the addition of L-glutamate.

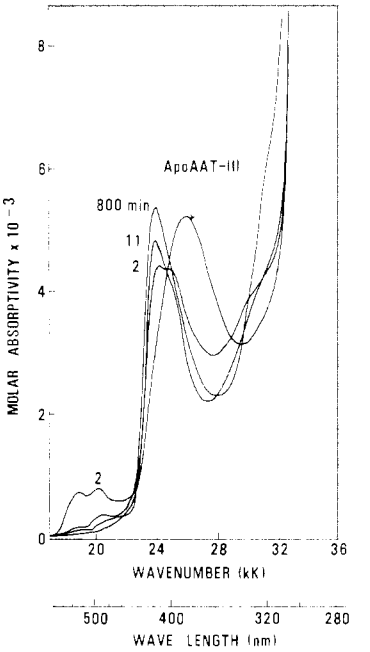
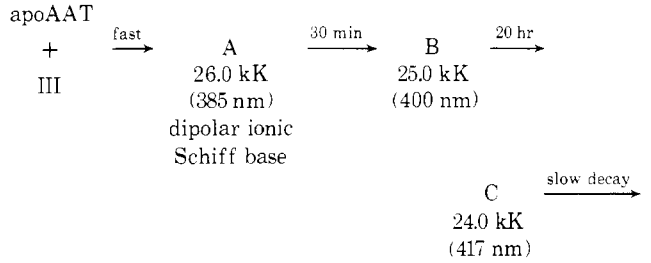


FIGURE 6: Treatment of the 26.0-kK (385 nm) form of apoAAT-III with DL-*erythro*-β-hydroxyaspartate. After apoAAT (103 μM) and compound III (72.9 μM) has been incubated at 27° in 0.1 M triethanolamine buffer (pH 8.3), DL-*erythro*-β-hydroxyaspartate (pH 8.3) was added. Final concentrations were: apoAAT, 101 μM; III, 71.5 μM; DL-*erythro*-β-hydroxyaspartate, 4.29 mM. The numbers are the times in minutes following the addition of DL-*erythro*-β-hydroxyaspartate.

1974).

The sequence of changes suggests the existence of three products A, B and C:



When the initial product (A) absorbing at 26.0 kK (385 nm) was treated with an excess amount of sodium borohydride, the absorption spectrum underwent a change yielding a band at about 29.5 kK (339 nm) and a weak band at 24.5 kK (408 nm). This further indicates that the initial product is a (dipolar ionic) Schiff base and that its reduction yields a secondary amine whose absorption at 29.5 kK corresponds to that of the reduced form (30.3 kK, 330 nm) of holoAAT. The difference of 0.8 kK in peak positions is accounted for by the double bond of III being conjugated to the pyridine nucleus. The small band at 24.5 kK (408 nm) is to be attributed to incomplete conversion of the Schiff base to the final product. Treatment of the 24.0-kK (417 nm) form (C) with either sodium borohydride or sodium cyanoborohydride had no effect on its absorption spectrum. This is another characteristic in common with the compound of pyridoxal 5′-sulfate and apoAAT.

When L-glutamate was added to complex A the 24.0-kK (417 nm) band appeared immediately as did another band at about 30 kK (333 nm). Though the latter decreased with time, the former changed little (Figure 5). Formation of the 24.0-kK species is thus remarkably enhanced by the presence of glutamate. This enhancement is inhibited by the presence of α-ketoglutarate. The intensity of the 24.0-kK band formed in the presence of glutamate is only one-half that in the absence of glutamate (compare Figures 3 and 5). The peak position of the higher energy band at 30 kK (333 nm) is appropriate for either a tetrahedral adduct at the 4′ position of the aldehyde (as is discussed later for nonenzymatic reactions) or for the corresponding pyridoxamine derivative. We favor the former explanation. In either case it appears that when half of the cofactor is converted to the 24.0-kK chromophore the other half is changed into a form absorbing at 30 kK (333 nm). The simplest explanation is that one subunit reacts to form the 24.0-kK chromophore and in so doing influences the other subunit to react differently. Still to be explained is the slow conversion of the 30.0-kK form into a species absorbing at still higher energies.

Complex A was likewise treated with DL-*erythro*-β-hydroxyaspartate (Figure 6), which is known to react with holoAAT in such a way that transamination is "locked" at the stage of the quinonoid intermediate yielding a band at 20.3 kK (492 nm). The formation of the 24.0-kK form was again enhanced but to a lesser extent than with glutamate. Most interesting is the appearance of two small transient bands in the "quinonoid" region immediately after the addition of *erythro*-β-hydroxyaspartate (Figure 6). One is at 19.0 kK (526 nm) and the other at 20.3 kK (493 nm). They disappear after approximately 30 min. The initial spectrum is also accompanied by a band at about 30 kK (333 nm), which decreases with time. The intensity of the final 24.0-kK peak is smaller than that of apoAAT-III but is greater than one-half of it.

At pH 9.8 the reaction of III and apoAAT proceeded, as at pH 8.3, to yield complex C. However, at pH 5.2 the final reaction product absorbs maximally at 25.2 kK (397 nm) and at pH 6.0 and pH 6.9 mixtures of the 25.2- and 24.0-

kK bands are present at equilibrium. The addition of an excess of sodium borohydride to the 25.2-kK (397 nm) form did not change the absorption spectrum. When the product formed at pH 5.2 or at pH 6.0 was dialyzed against 0.01 *M* triethanolamine buffer (pH 8.3) the absorption spectrum had a maximum at 24.0 kK. Conversely, when the 24.0-kK product (C) at pH 8.3 was dialyzed against 0.02 *M* acetate buffer (pH 5.0), the peak shifted to 25.2 kK. After dialysis against 0.01 *M* triethanolamine buffer (pH 8.3) it shifted back to 24.0 kK. These results suggest that the 24.0-kK form at higher pH and the 25.2-kK form at lower pH are two different ionic forms of complex C.

Denaturation of ApoAAT-III Complex. When complex C at pH 8.3 was denatured by heating at 100° for 5 min, the supernatant showed no absorption below 35 kK (above 286 nm). The spectrum of the precipitate at pH 8.5 in saturated urea had a maximum at 24.1 kK. The 24.0-kK form at pH 8.3 was also denatured with trichloroacetic acid with similar results. The precipitate in saturated urea at pH 6.9 had an absorption maximum at 25.0 kK (400 nm), which shifted to 24.0 kK (417 nm) when the pH was adjusted to 9.7. Reprecipitation of the protein with trichloroacetic acid did not release the chromophore.

When a solution containing complex C at pH 8.3 was brought to pH 12.1 by adding 4 *N* sodium hydroxide, the 24.0-kK band decreased rapidly and further gradual changes occurred to form a complex pattern. The CD showed no distinct band suggesting that the enzyme had been denatured. Then the protein was precipitated with a few drops of 50% trichloroacetic acid. The precipitate and the supernatant were separated by centrifugation. Again the spectrum of the supernatant showed no band from 18 to 35 kK (556–286 nm), whereas the precipitate redissolved in saturated urea with pH adjusted to 12.0 showed an absorption maximum at about 24.5 kK (408 hm).

Thus the chromophore stayed with the protein during denaturation whether by heat, acid, or base. The chromophore remained intact during the heat and acid denaturations. Reversible interconversion between the high and low pH spectral forms was still observed in the denatured enzyme.

Nonenzymatic Model Reactions. In an attempt to elucidate the nature of the apoAAT-III reaction, various nonenzymatic reactions were investigated. Like pyridoxal 5′-sulfate, III was found to react with certain amino acids and diamines, to form products with spectra analogous to that of complex C. These compounds include arginine, α-amino-γ-guanidinobutyric acid, ornithine, cysteine, α,γ-diaminobutyric acid, ethylenediamine, and 1,3-diaminopropane. They all react with III in the pH region from 9 to 10 and yield final products with narrow bands in 24.0–24.4-kK region. The cleanest reactions observed were with diaminobutyrate (Figure 7) and with diaminopropane. The final products in both cases have narrow absorption bands at 24.4 kK (410 nm), are difficult to reduce with sodium borohydride, and have a bright blue fluorescence similar to that of C. The position and the shape of the 24.4-kK bands resemble those of C and of the product of pyridoxal 5′-sulfate with β-substituted amines (Yang et al., 1974).

1,3-Diaminopropane is known to react at basic pH with pyridoxal-P to form a hexahydropyrimidine derivative (O'Leary, 1971) absorbing at 32.1 kK (312 nm). By analogy, the initial products in the reactions of III with diaminopropane (29.9 kK) and with diaminobutyrate (30.0 kK, 333 nm) are hexahydropyrimidine derivatives. The peak positions agree with that of the pyridoxal-P-diaminopropane

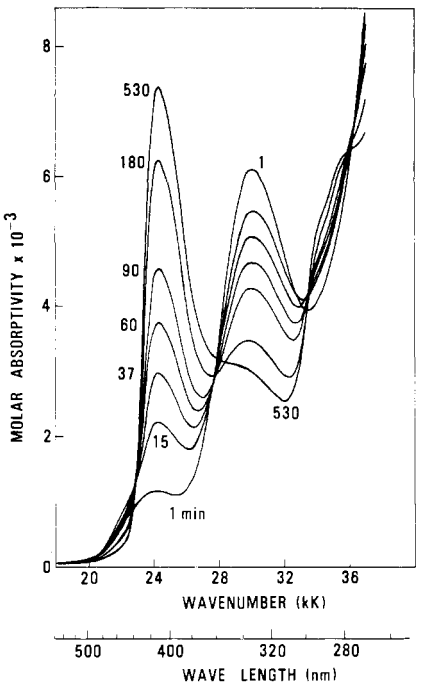
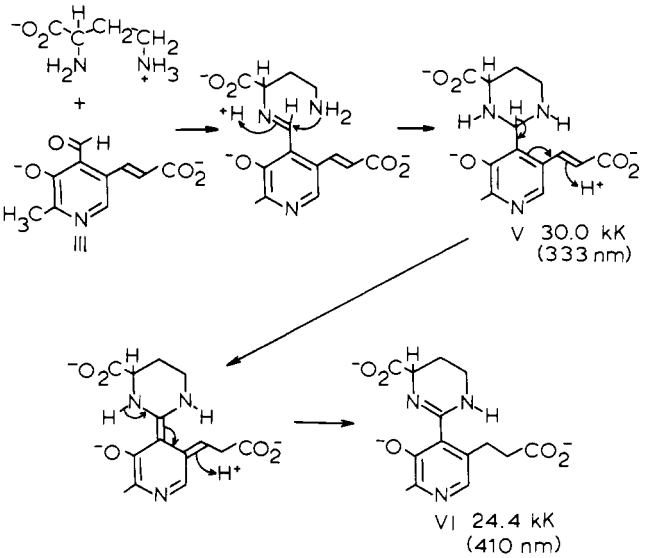


FIGURE 7: The reaction of compound III (159 μ*M*) with α,γ-diaminobutyric acid (39.3 μ*M*) at 27° in 0.2 *M* bicarbonate buffer (pH 9.7). The numbers designate the times in minutes following the mixing of the two compounds.

Scheme I



product if the extra double bond in III is taken into account. Based on the spectra change and the analogy with the reactions of pyridoxal 5′-sulfate, a tentative reaction pathway was proposed (Scheme I). The initial hexahydropyrimidine derivative (V) undergoes a two-stage tautomerization sequence leading to the cyclic Schiff base (VI) which absorbs at 24.4 kK (410 nm) as the final product. This pathway was confirmed by following the reaction with NMR. Figure 8 shows the change in the 60-MHz NMR spectrum of the reaction of diaminobutyrate with III. The spectrum obtained a few minutes after the reaction had started shows the formation of the hexahydropyrimidine derivative (V) characterized by the appearance of the 4′-H. It is clear that as the reaction went on, the vinyl protons as well as the 4′-proton totally disappeared. The appearance of new peaks corre-

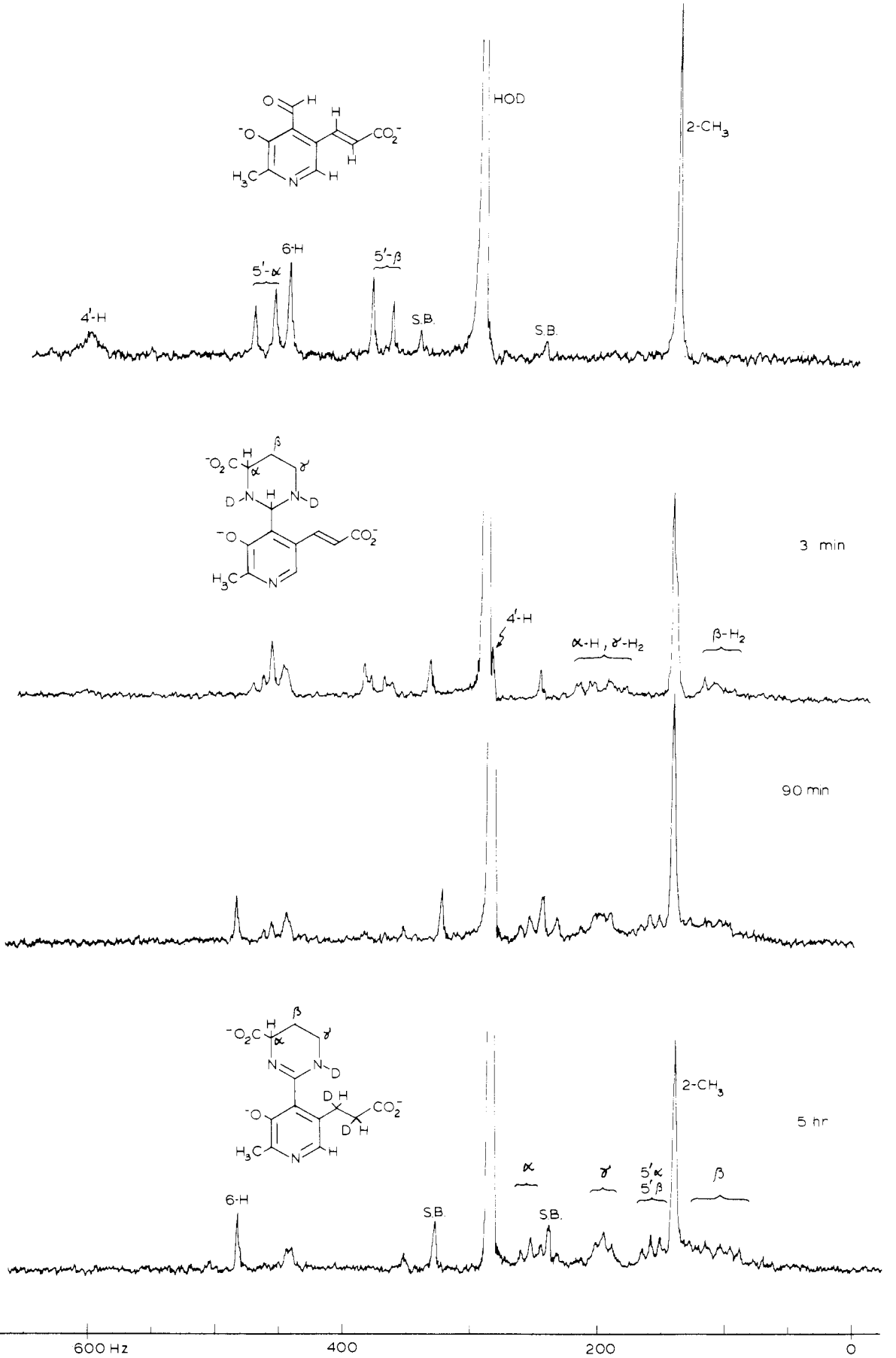
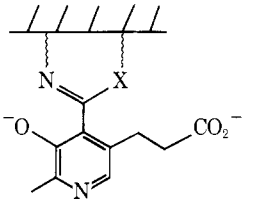


FIGURE 8: The 60-MHz NMR spectrum of a molar (0.25 *M*) solution of compounds III and 2,4-diaminobutyrate in 4% NaOD/D_2O with tetramethylsilane as an external standard. The top curve is the spectrum of compound III before the addition of diaminobutyrate. The final pD of the mixture was 12.7. The assignments for the initial cyclic adduct (V) and for the final product (VI) are indicated.

sponding to the 5α-H and 5β-H and to the α-H, β-H_2, and γ-H_2 supports the structure VI for the final product. The assignment of the 5α-H and 5β-H is based upon the NMR spectra of 5′-deoxy-5′-carboxymethylpyridoxal (Iwata and Metzler, 1967) and 5′-deoxy-5′-carboxymethylpyridoxine (Tomita et al., 1966). The correspondence of the final NMR spectrum in Figure 8 (bottom curve) to the 24.4-kK (410 nm) product was proved by taking an aliquot from the NMR sample and measuring its absorption spectrum.

Discussion

The mechanism proposed for the reaction of the vinylcarboxylic acid analogue of pyridoxal-P (III) and α,γ-diaminobutyric acid will not be fully established until the product is isolated and characterized in detail. However, the mechanism is consistent with the observed changes in absorption and NMR spectra and with the reactivity of pyridoxal 5′-sulfate with β-substituted amines. Judging from the resemblance of the product of the model reaction and that of apoAAT-III, we propose structure VII for the 24.0-kK



VII

form (complex C) of the apoAAT-III product. It is presumed to be formed from the initial Schiff base with the ϵ-amino group of Lys-258 through a pathway similar to that in Scheme I with an X group from the enzyme replacing the γ-amino group of α,γ-diaminobutyrate. The apoenzyme is covalently modified through the X group. It is quite probable that the final chromophore still occupies the active site, since the initial product is a "normal" Schiff base and the III-modified apoenzyme cannot at all regain its activity by the addition of pyridoxal-P. Thus a new aspect of the active site chemistry may be revealed if the X group is identified. Tryptic digestion and identification of peptide fragments containing group X are in progress. It is to be emphasized here that the reactions of apoAAT with III and with pyridoxal 5′-sulfate have so many similarities that the X group is probably identical in both cases.

Pyridoxal-P has been used to modify various proteins (Schnackerz and Noltmann, 1971; Raetz and Auld, 1972; Benesch et al., 1974). Presumably III will also interact with other proteins and may possibly label some of them irreversibly. Thus, it may be a useful protein-modifying reagent.

From the spectral data of Table I, making use of the estimated molar areas given there, it is possible to estimate microscopic dissociation constants for the phenolic hydroxyl group and the pyridinium group. We find the pK_a for dissociation of the pyridinium group of the dipolar ionic form of alcohol IV as 8.8, just 0.1 unit lower than that of pyridoxine. On the other hand, the same constant for dissociation of the dipolar ion of III is 6.8, 1.1 unit less than that of the corresponding group in 5′-deoxypyridoxal for which the pK_a is 7.7. By comparison, the same constant for the saturated analogue 5′-deoxy-5′-carboxymethylpyridoxal is 7.9, for pyridoxal-P it is 8.1, and for pyridoxal 5′-sulfate, 7.1. Effects on the microscopic dissociation constants of the phenolic hydroxyl parallel the above almost exactly. It is of interest that both the 5′-CH_2-O-SO_3^- and —CH=CH-COO^- groups appear to exert a strong electron-withdrawing effect in the aldehydes but have much less effect on the ring in the corresponding 4′-alcohols. The electronic properties of these two substituents may provide the explanation for the ease of elimination of sulfate in model reactions (Yang et al., 1974) and for the novel reactions of III described in this report.

The positions of the absorption bands of both III and IV are shifted bathochromically from those of related compounds lacking the double bond in the side chain. For example, the peak position of the dipolar ion of III is 0.6 kK lower than that of I or II (Harris et al., 1975) and the position of the dipolar ion peak of IV is 1.2 kK lower than that of pyridoxine. Most striking is the 2.5-kK bathochromic shift for the anion of IV as compared with pyridoxine (Metzler et al., 1973). It would appear that interaction between the ring (as an electron donor) and the vinylcarboxylate group (as an electron acceptor) must be especially strong in the excited state of the anion of IV.

The existence of two transient bands, at 19.0 kK (526 nm) and 20.3 kK (493 nm), of a type usually attributed to quinonoid intermediates suggests the possibility that these represent two different conformational states of the coenzyme (and of the protein, Ivanov and Karpeisky, 1969). In the 19.0-kK form the side chain would be coplanar with the ring while in the 20.3-kK form it would be turned out of plane.

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The Isolation and Partial Characterization of Diphosphoglycerate Mutase from Human Erythrocytes†

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ABSTRACT: Diphosphoglycerate mutase has been purified to homogeneity from outdated human erythrocytes. The native enzyme has a molecular weight of 57 000 as determined by equilibrium centrifugation and exclusion chromatography. Disc gel electrophoresis in the presence of sodium dodecyl sulfate yields a single protein band with a molecular weight of about 26 500, indicating that diphosphoglycerate mutase is comprised of two subunits of similar mass. The enzyme exhibits the following intrinsic activities: diphosphoglycerate mutase, monophosphoglycerate mutase, and 2,3-diphosphoglycerate phosphatase. The latter activity is enhanced in the presence of either organic or inorganic anions. Glycolate-2-P, particularly, has a profound activating effect. Nonspecific phosphatase and enolase activities are absent. The enzyme has an extinction coefficient at 280 nm of 1.65 cm^2/mg. The amino acid composition of the homogeneous protein has been determined.

Rapoport and Luebering (1950, 1951) were the first investigators to propose that 2,3-diphosphoglycerate (2,3-DPG)[1] levels within the erythrocyte are maintained by two specific enzymes: bisphosphoglyceromutase (EC 2.7.5.4): diphosphoglycerate mutase, DPGM, and bisphosphoglycerate phosphatase (EC 3.1.3.13): diphosphoglycerate phosphatase, DPGP. After the physiologically important observation that the presence of 2,3-DPG profoundly influences the complexation between oxygen and hemoglobin (Chanutin and Curnish, 1967; Benesch et al., 1968), several attempts have been made to isolate each of the above red cell enzymes (Sasaki et al., 1975; Rose and Whalen, 1973; de Verdier and Groth, 1973; Harkness et al., 1970; Rose and Liebowitz, 1970). Recently, however, several reports have suggested that intracellular 2,3-DPG concentrations may be regulated by one protein, DPGM, which has been shown to manifest both mutase and phosphatase activity (Kappel et al., 1975; Hass and Miller, 1975; Sasaki et al., 1975; Rosa et al., 1973). If this is the case, the original Rapoport–Luebering proposal is incorrect; consequently, a separate DPGP is probably nonexistent.

In order to test the validity of the above hypothesis and to study the multifunctionality of DPGM, we have devised a relatively simple scheme for the isolation of homogeneous diphosphoglycerate mutase from outdated human erythrocytes.[2] Previously described methods for the purification of DPGM have resorted to the use of isoelectric focusing (Rose and Whalen, 1973; Sasaki et al., 1975), a technique, which in its current state of development, renders impractical the isolation of large quantities of enzyme. Our procedure, on the other hand, is limited solely by the availability of biological material.

In addition to describing a process for the purification of diphosphoglycerate mutase, this report contains data which both corroborate and supplement certain physicochemical characteristics of the DPGM molecule.

Experimental Procedures

Materials. Outdated human erythrocytes of various blood types were obtained from The Hershey Medical Center blood bank. NAD^+, carboxymethylcellulose, dithiothreitol, the diethyl acetal (barium salt) of DL-glyceraldehyde-

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[1] Abbreviations used are: 2,3-DPG, 2,3-diphosphoglycerate; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase; 3-PGA, D-glycerate-3-P; 2-PGA, D-glycerate-2-P; MPGM, monophosphoglycerate mutase.

[2] This report is an expansion of a preliminary communication by Kappel et al. (1975).